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(71) Applicant *(for all designated States except US)*: FORSKNINGSSTIFTELSEN DET NORSKE RADIUMHOSPITAL [NO/NO]; Montebello, N-0310 Oslo 3 (NO).

(71)(72) Applicant and Inventor: OLSNES, Sjur [NO/NO]; Vestveien 8, N-0284 Oslo 2 (NO).

(74) Agent: TANDBERGS PATENTKONTOR AS; Uranienborg terrasse 19, N-0351 Oslo 3 (NO).

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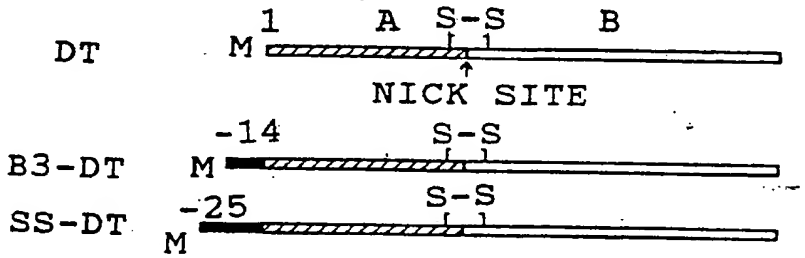
(54) Title: METHOD OF INTRODUCING A PEPTIDE INTO THE CYTOSOL



(57) Abstract

A method of introducing a peptide into the cytosol by linking the peptide to a bacterial or plant toxin, or a mutant thereof. A method of preparing a vaccine by linking a peptide to a bacterial or plant toxin, or a mutant thereof to translocate the peptide into the cytosol for subsequent presentation at the cell surface by Class I MHC antigens to elicit a Class I restricted immune response and to expand the relevant population of CD8+ T-lymphocytes. Vaccines produced by said method and the use thereof against viruses, intracellular bacteria and parasites, and against molecules associated with malignancies.

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Method of introducing a peptide into the cytosol

Field of the Invention

The present invention is directed to a method of introducing a peptide into the cytosol, and more specifically to a novel principle in vaccine production against viruses, intracellular parasites and bacteria and against malignant cells.

Background of the Invention

In the protection against pathogenic organisms and in their elimination antigen presentation by major histocompatibility antigens (MHC) of class I plays an important role. Cytotoxic T-lymphocytes recognize cells that express foreign or unusual antigens on their surface and destroy the cells, which is important to eliminate an infection. The same mechanism is operating in the elimination of malignant cells. Antigen presentation by Class I MHC requires that the antigen to be presented is found in the cytosol or in the endoplasmic reticulum (Germain, R.N. Nature 322, 687-689 (1986)). Externally added polypeptides therefore do normally not elicit a class I response. However, if the antigen is artificially introduced into the cytosol, presentation by MHC Class I may occur (Moore, M.W., Carbone, F.R. & Bevan, M.J. Cell 54, 777-785 (1988)). The common way today to immunize against such structures is to use attenuated live viruses that are able to enter cells and replicate such that the peptides in question are formed in the cells and can be presented at the cell surface. In this way the population of the relevant cytotoxic $CD8^+$ cells is expanded and upon later exposure to the corresponding virulant virus strain, the organism has an immune protection. The problems with this approach are partly due to the fact that the attenuated viruses may sometimes revert to virulence and partly to the problems of making attenuated viruses in many cases. Convenient and non-damaging methods to introduce into the cytosol foreign peptides, such as viral antigens, could therefore be useful for vaccine purposes to expand the relevant population of $CD8^+$ MHC Class I restricted cytotoxic T-lymphocytes.

The only established examples of external proteins that enter the cytosol are certain bacterial and plant toxins, such as diphtheria toxin, Pseudomonas aeruginosa exotoxin A, ricin, abrin, viscumin, modeccin, Shigella toxin, cholera toxin, pertussis toxin (Olsnes, S. & Sandvig, K. In: "**Immunotoxins**" (A.E. Frankel, ed.), Kluwer Academic Publishers, Boston 1988, pp. 39-73; Olsnes, S. & Sandvig, K. In "**Receptor-mediated endocytosis**" (I. Pastan & M.C. Willingham, eds.), Plenum Publ. Corp., 1985, pp. 195-234). Toxins of this group enter the cytosol where they carry out enzymatic reactions that are deleterious to the cell or to the organism. By gene manipulations it is possible to form toxin molecules that are of very low toxicity (Barbieri, J.T. & Collier, R.J. Infect. Immun. 55, 1647-1651 (1987)). If the toxins were able to carry into cells additional peptide material, such non-toxic mutants could be useful for vaccine purposes to carry into the cytosol antigenic peptides (Cerundolo et al. Nature 345, 449 (1990)) that can be presented by Class I MHC antigens. Such antigenic sequences can be obtained from a number of viruses, bacteria and parasites, and it is also possible to derive such structures from certain malignant cells.

It is an object of the present invention to provide a mechanism of translocating antigenic peptide sequences to the cytosol in a safe way to expand the population of cytotoxic T-lymphocytes that are able to react with the corresponding antigen and eliminate those cells that are presenting the antigenic peptides. Although the entry mechanism for the different toxins mentioned above is in principle the same, it has been worked out in most detail in the case of diphtheria toxin. This is the toxin we have used in most of our studies in connection with this application.

Summary of the Invention

We here demonstrate that an essentially non-toxic mutant of diphtheria toxin is able to translocate to the cytosol oligopeptides linked to its N-terminal end. The peptides we have studied are sufficiently different in sequence to allow the conclusion that a wide variety of peptides can be

carried into the cells in the same way.

Thus, the present invention relates to a method of introducing a peptide into the cytosol by linking the peptide to a bacterial or plant toxin, or a mutant thereof. Further, the present invention relates to a method of preparing a vaccine by linking a peptide to a bacterial or plant toxin, or a mutant thereof to translocate the peptide into the cytosol for subsequent presentation at the cell surface by Class I MHC antigens to elicit a Class I restricted immune response and to expand the relevant population of $CD8^+$ T-lymphocytes. Also, the present invention relates to vaccines which have been produced by the above-mentioned method, as well as the use of such vaccines against viruses, intracellular bacteria and parasites, and against molecules associated with malignancies.

Figure Legends

FIG. 1. N-terminal extensions of diphtheria toxin.

A. The coding region of the diphtheria toxin gene carrying a triple mutation changing Glu^{148} to Ser, and where Gly^1 was replaced by initiator Met placed behind a T3 promotor to give pBD-1S (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.: EMBO J. 8, 2843-2848 (1989)). To obtain pB-B3-D1, pBD-1 was cleaved with NcoI, and an oligonucleotide encoding the oligopeptide MGVDEYNEMPMPVN (referred to as B3) was inserted. pGD-2 encodes diphtheria toxin with its natural signal sequence, MSRKLFASILIGALLGIGAPPSAHA (referred to as ss), after an SP6 promotor. The plasmid was obtained by digesting pGD-1 (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.: EMBO J. 8, 2843-2848 (1989)) with HindIII and PstI, removing the overhangs with S_1-nuclease and religating to form pGD-2.

B. The genes were transcribed in vitro and the mRNAs obtained were translated in rabbit reticulocyte lysate systems in the presence of [^{35}S]methionine (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.: EMBO J. 8, 2843-2848 (1989)). To remove reducing agents and to allow disulfide bridges to be formed, the translation mixture was dialyzed over night against PBS (0.14 M NaCl, 10 mM Na-phosphate, pH 7.4), and then for 4 h against Hepes medium (Dulbecco-modified Eagles

medium wherein the bicarbonate had been replaced by 20 mM Hepes, pH 7.4). An aliquot of each sample was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing conditions (Olsnes, S. & Eiklid, K. J. Biol. Chem. 255, 284-289 (1980)). In some cases the translation product was treated with protein A-Sepharose (Pharmacia, Sweden), which had previously been incubated with rabbit anti-B3 antiserum (lanes 3 and 4) or anti-ricin (lane 5). The adsorbed material was analyzed by SDS-PAGE. DT, translation product from pBD-1; B3-DT, translation product from pB-B3-D1; ss-DT, translation product from pGD-2.

FIG. 2. Translocation to the cytosol of A-fragment with N-terminally added B3 oligopeptide. pBD-1 and pB-B3-D1 were transcribed and translated in vitro. The corresponding translation products (DT and B3-DT) were added to Vero cells growing as monolayers in 24-well microtiter plates and kept at 24°C for 20 min in the presence of 10 µM monensin (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.: EMBO J. **8**, 2843-2848 (1989)). The cells were washed twice with Hepes medium and subsequently treated with 0.4 µg/ml TPCK (N-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin in Hepes medium containing 10 µM monensin for 5 min at 20°C. The cells were washed and exposed to Hepes medium, pH 4.8, containing 10 mM Na-gluconate to increase the buffering capacity at the low pH. After 2 min at 37°C, the cells were washed with Hepes medium, pH 7.4, and then treated with 3 mg/ml pronase in Hepes medium, pH 7.4, containing 10 µM monensin for 5 min at 37°C. The cells, which were detached from the plastic by the treatment, were recovered by centrifugation and washed once with Hepes medium containing 1 mM NEM (N-ethyl maleimide) and 1 mM PMSF (phenylmethylsulfonyl fluoride). In some cases, (lanes 1-3 and 8-10) the cells were lysed with Triton X-100 in phosphate buffered saline containing 1 mM PMSF and 1 mM NEM, nuclei were removed by centrifugation and the protein in the supernatant fraction was precipitated with 10% (w/v) trichloroacetic acid or immunoprecipitated with anti-B3 antibodies adsorbed to protein A-Sepharose. In other cases (lanes 4-7) the cells were treated with 50 µg/ml saponin in PBS containing 1 mM PMSF and

1 mM NEM to release translocated A-fragment, and then the proteins both in the pellet and in the supernatant fractions were precipitated with trichloroacetic acid. In all cases the precipitated material was analyzed by SDS-PAGE (13.5% gel) under non-reducing conditions.

FIG.3. Translocation to the cytosol of diphtheria toxin with signal sequence. Lanes 1-4: ^{125}I-labelled natural toxin (wt-DT, lane 1) and in vitro translated pGD-2 ([^{35}S]methionine labelled toxin with signal sequence, ss-DT) were bound to Vero cells and nicked on the cells (lanes 1 and 2). In lane 3 the cells were treated as in lane 2, except that 6 times more translation product was used and the cells were then exposed to pH 4.8 and pronase as in Fig. 2. The cells were lysed with Triton X-100 and the nuclei were removed. The supernatants were incubated with protein A-sepharose that had been pre-incubated with rabbit anti-diphtheria toxin serum. The adsorbed material was analyzed by reducing (lanes 1 and 2) or non-reducing (lanes 3 and 4) SDS-PAGE (10% gel). In lane 4 the pronase-treated cells were treated with 50 µg/ml saponin and the material released to the medium was analyzed directly. Lanes 5-12: Translation products from pBD-1 (DT) and pGD-2 (ss-DT) were bound to Vero cells, nicked, exposed to pH 4.8 and then treated with pronase. The lysed cells were either analyzed with non-reducing SDS-PAGE (15% gel) directly (lanes 5-8) or they were treated with saponin and the membrane pellets (lanes 9 and 10) and the supernatant fractions (lanes 11 and 12) were analyzed separately.

Detailed Description

Diphtheria toxin is synthesized by pathogenic strains of Corynebacterium diphtheriae as a single chain polypeptide. The protein is easily split ("nicked") at a trypsin-sensitive site to yield two disulfide-linked fragments, A and B (Pappenheimer, A.M., Jr. Annu. Rev. Biochem. 46, 69-94 (1977)).

The B-fragment (37 kD) binds to cell surface receptors, whereas the A-fragment (21 kD) is an enzyme that is translocated to the cytosol where it inactivates elongation factor 2 by ADP-ribosylation and thus blocks protein synthesis (Van Ness, B.G., Hovard, J.B. & Bodley, J.W. J. Biol. Chem. 255,

10710-10716 (1980)). The translocation, which normally occurs across the limiting membrane of endosomes, is triggered by the low pH in the acidic vesicles (Draper, R.K. & Simon, M.I. J. Cell Biol. 87, 849-854 (1980); Sandvig, K. & Olsnes, S. J. Cell Biol. 87, 828-832 (1980)). When cells with surface-bound toxin are exposed to acidic medium, translocation occurs from the cell surface (Sandvig, K. & Olsnes, S. J. Biol. Chem. 256, 9068-9076 (1981)). We have in the presented examples used this artificial system, because it enables us to distinguish between translocated and non-translocated material (Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 262, 10339-10345 (1987); Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 263, 2518-2525 (1988)).

To avoid toxic effect on the cells by the diphtheria toxin vector, a mutant toxin was used which contains a triple mutation changing Glu^{148}, which is located in the enzymatically active site of the toxin, to Ser (Barbieri, J. T. & Collier, R.J. Infect. Immun. 55, 1647-1651 (1987)). The modified toxin has strongly reduced toxicity.

Examples

We used two variants of the mutated toxin gene, one without (pBD-1) (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.: EMBO J. 8, 2843-2848 (1989)), and one with (pGD-2) the natural 25 amino acids signal sequence (Fig. 1A). In one case, a foreign oligopeptide, termed B3, was linked to the N-terminal end of the toxin to yield the plasmid pB-B3-D1.

The constructs, which were placed behind T3 or SP6 RNA-polymerase promotors, were transcribed and translated in vitro (McGill, S., Stenmark, H., Sandvig, K. & Olsnes, S.:EMBO J. 8, 2843-2848 (1989)). In each case a major band corresponding to the full-length protein and only traces of material of lower molecular weights were obtained (Fig. 1B). Toxin with signal sequence (lane 7) or with B3 (lane 1) migrated, as expected, slightly more slowly than toxin as such (lanes 2 and 6). Furthermore, toxin with B3 was selectively precipitated with anti-B3 (lane 4), but not with a control serum (lane 5). Toxin without B3 was not precipitated with anti-B3 (lane 3).

The dialyzed translation products were bound to Vero

cells, nicked on the cells with low concentrations of trypsin, and then the cells were exposed to pH 4.8. Under these conditions part of the bound toxin was translocated to the cytosol and thereby became shielded against pronase added to the medium (Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 263, 2518-2525 (1988)). In the case of diphtheria toxin as such, two fragments (MW 21 kD and 25 kD) were protected under these conditions (Fig. 2, lane 1), corresponding to the whole A-fragment (21 kD) and part of the B-fragment (25 kD out of total 37 kD). The interfragment disulfide was reduced, apparently upon exposure to the cytosol (Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 262, 10339-10345 (1987)).

Example 1

When the same experiment was carried out with toxin containing B3, two major fragments (25 kD and 22.5 kD) were protected in addition to small amounts of 21 kD fragment (lane 2). The latter probably represents A-fragment where B3 had been cleaved off. When the exposure to low pH was omitted, no fragments were protected (lane 3). The 22.5 kD fragment was precipitated by anti-B3 (lane 9), but not with preimmune serum (lane 10). Protected A-fragment without the oligopeptide was not precipitated with anti-B3 (lane 8). The apparently higher amount of protected A-fragment with B3 is due to more radioactivity incorporated, as B3 contains 3 methionines and the A-fragment alone 5.

When cells with translocated diphtheria toxin are treated with low concentration of saponin allowing cytoplasmic marker enzymes to leak out of the cells without dissolving the membranes, the translocated A-fragment is released into the medium, whereas the B-fragment-derived 25 kD polypeptide remains associated with the membrane fraction (Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 263, 2518-2525 (1988); Moskaug, J.Ø., Sletten, K., Sandvig, K. & Olsnes, S. J. Biol. Chem. 264, 15709-15713 (1989); Moskaug, J.Ø., Sandvig, K. & Olsnes, S. J. Biol. Chem. 264, 11367-11372 (1989)). This indicates that the translocated A-fragment is free in the cytosol, whereas the 25 kD polypeptide is inserted into the membrane.

Also most of the A-fragment containing B3 was released with saponin (lane 7) in the same way as normal A-fragment (lane 6), whereas the 25 kD fragment was associated with the membranes (lanes 4 and 5). Therefore, it appears that diphtheria toxin is able to translocate B3 (14 amino acids) to the cytosol.

Example 2

To test if also a larger oligopeptide could be translocated, we chose toxin carrying its normal signal sequence (25 amino acids). As shown in Fig. 3, lane 2, this protein was nicked by trypsin into a 23.5 kD A-fragment and a 37 kD B-fragment. (In this experiment the toxin was only partially nicked. Partially nicked ^{125}I-labelled natural toxin is shown for comparison in lane 1). When the toxin with signal sequence was bound to cells, nicked, and then exposed to pH 4.8, two fragments (23.5 kD and 25 kD) were protected against pronase (lane 8). Protected A-fragment with uncleaved signal sequence is also shown in lane 3, where the material was precipitated with an anti-diphtheria toxin serum which binds the whole toxin, the A-fragment, as well as whole B-fragment (see lanes 1 and 2), but not the 25 kD-fragment. When the pronase-treated cells were treated with saponin, the extended A-fragment was released to the medium (lanes 4 and 12), whereas the 25 kD fragment remained in the membrane fraction (lane 10).

Claims

1. A method of introducing a peptide into the cytosol, characterized by linking the peptide to a bacterial or plant toxin, or a mutant thereof.

2. A method of preparing a vaccine, characterized by linking a peptide to a bacterial or plant toxin, or a mutant thereof to translocate the peptide into the cytosol for subsequent presentation at the cell surface by Class I MHC antigens to elicit a Class I restricted immune response and to expand the relevant population of $CD8^+$ T-lymphocytes.

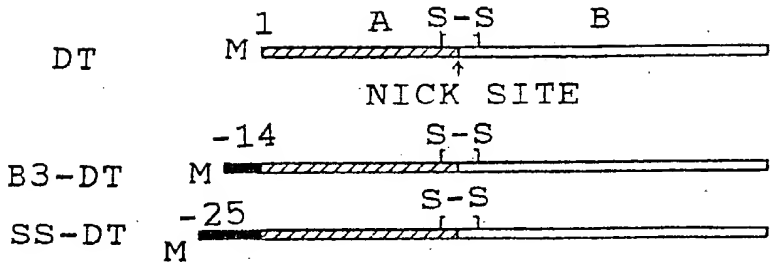
3. The method according to claims 1 or 2, characterized by using a mutant of a bacterial or plant toxin which has been manipulated in such a way that it has lost its toxicity without having lost the ability to enter the cytosol and to carry additional peptide material into the cytosol.

4. The method according to claims 1 or 2-3, characterized by using a non-toxic mutant of diphtheria toxin or a related toxin such as ricin, abrin, modeccin, viscumin, volkensin, Pseudomonas aeruginosa exotoxin A, Shigella toxin, cholera toxin, E. coli heat labile toxin or pertussis toxin.

5. The method according to claims 1 or 2-4, characterized by using a non-toxic mutant of diphtheria toxin.

6. A vaccine, characterized by having been produced by a method according to claims 2-5.

7. The use of a vaccine according to claim 6 against viruses, intracellular bacteria and parasites, and against molecules associated with malignancies.


DT
M
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A
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B
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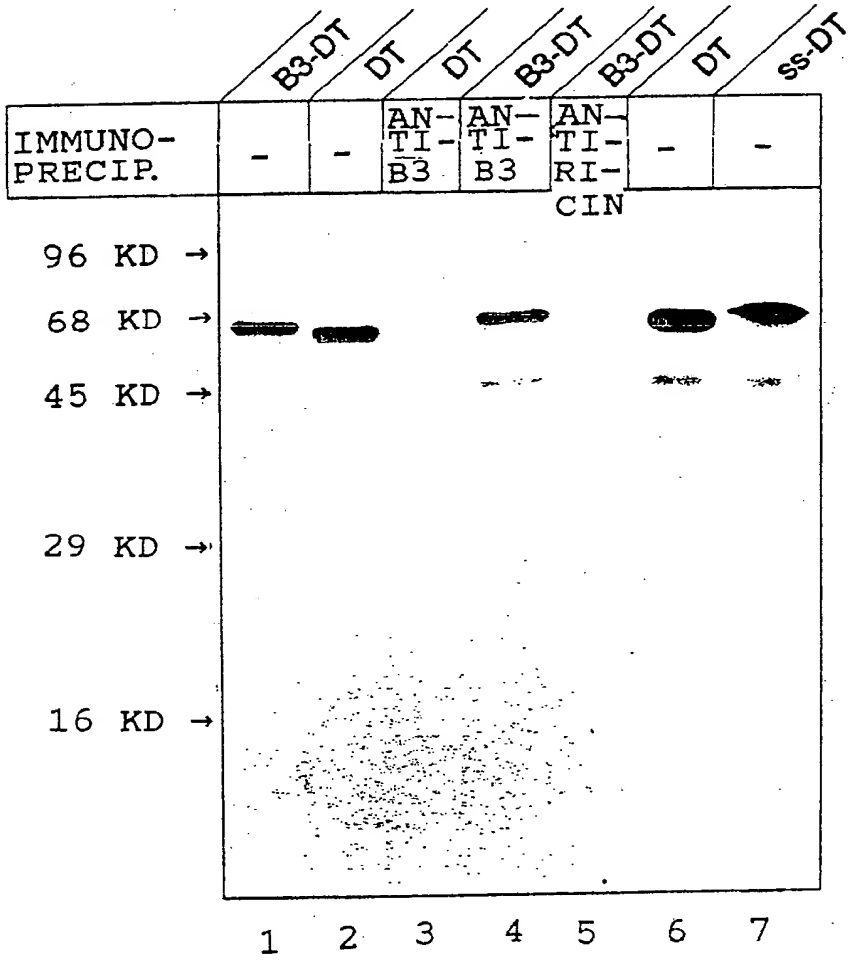
S-S
SS-DT
M
-25
S-S

FIG. 1 A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DT
B3-DT
B3-DT
DT
ss-DT
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-
-
ANTI-B3
ANTI-B3
ANTI-RICIN
-
-
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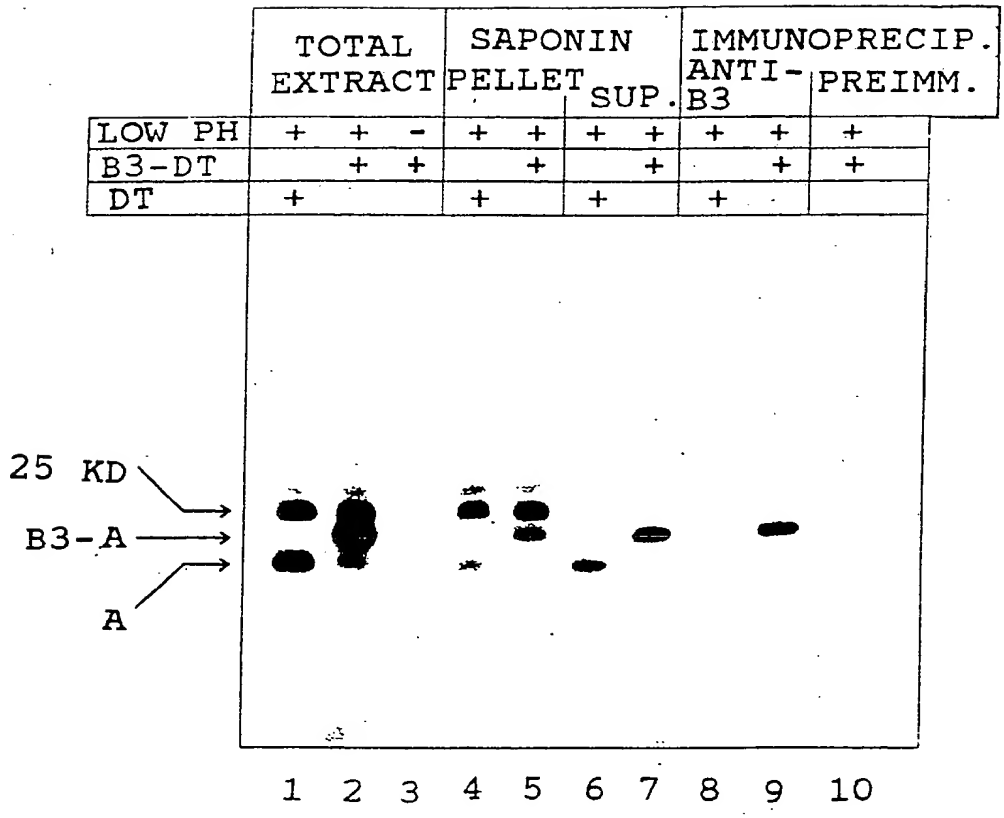
3
4
5
6
7

FIG. 1 B


TOTAL EXTRACT
SAPONIN PELLET
SUP.
IMMUNOPRECIP. ANTI-B3
PREIMM.
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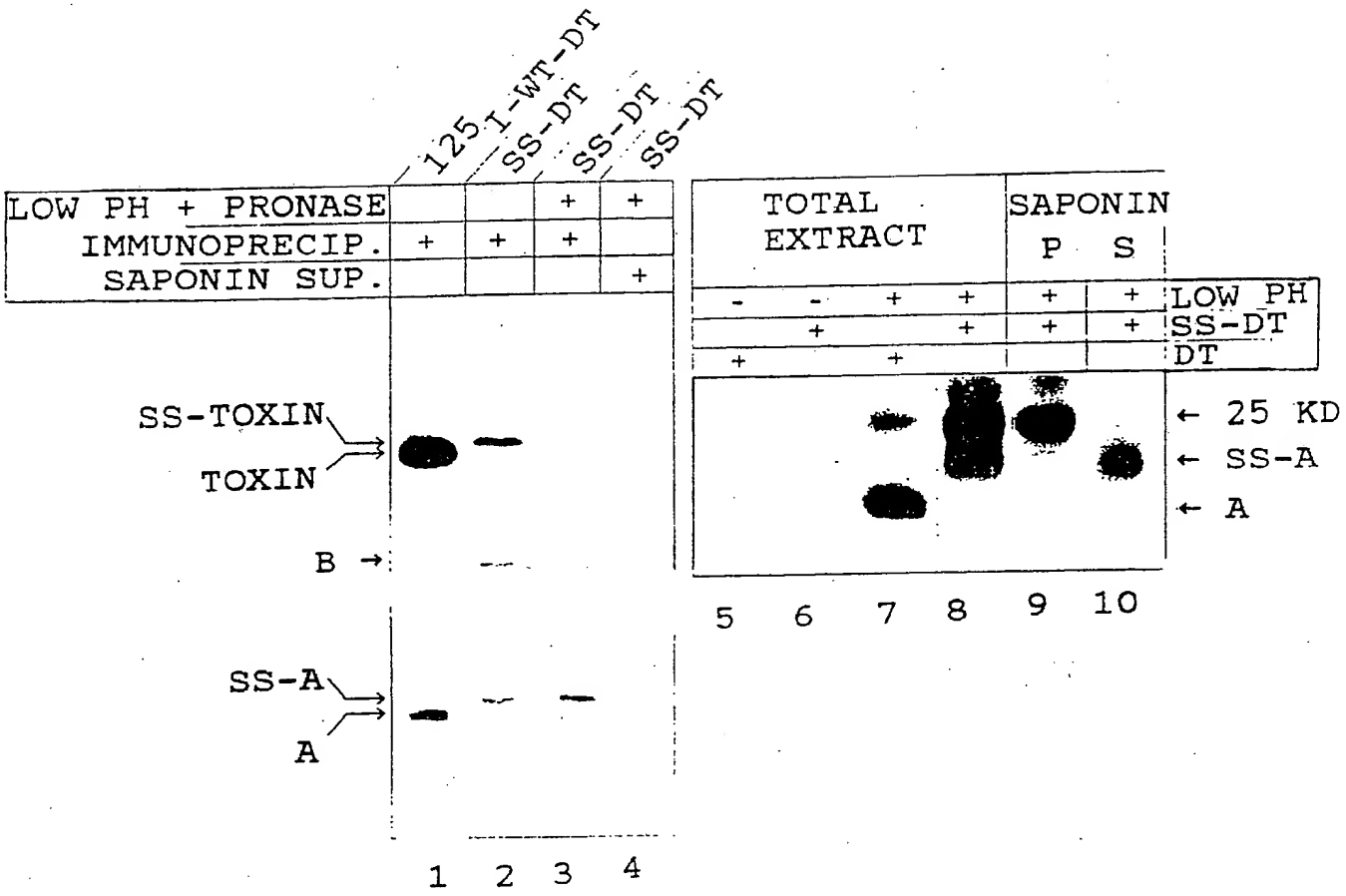
B3-DT
DT
25 KD
B3-A
A
1 2 3 4 5 6 7 8 9 10

FIG. 2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SS-DT
SS-DT
SS-DT
LOW PH + PRONASE
IMMUNOPRECIP.
SAPONIN SUP.
TOTAL EXTRACT
SAPONIN
P S
LOW PH
SS-DT
DT
SS-TOXIN
TOXIN
B
SS-A
A
← 25 KD
← SS-A
← A
1 2 3 4
5 6 7 8 9 10

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/NO 91/00093

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 39/385, C 12 N 15/62, A 61 K 47/48, C 07 K 13/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 12 N; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	Dialog Information Services, File 155, Medline 67-91, Dialog accession no. 07731463, Stenmark H et al: "Peptides fused to the amino-terminal end of diphtheria toxin are translocated to the cytosol", & J Cell Biol (UNITED STATES) Jun 1991, 113 (5) p1025-32 --	1-7
E	WO, A1, 9109871 (SERAGEN INCORPORATED) 11 July 1991, see for example claim 4 --	1-7
X	WO, A1, 9003437 (L'UNIVERSITE DE L'ETAT A LIEGE) 5 April 1990, see the whole document --	1-4,6-7

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22nd October 1991	1991 -10- 28
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Mikael Bergstrand / Yvonne Siösteen

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	EP, A2, 0332174 (THE UNIVERSITY OF WYOMING) 13 September 1989, see the whole document	1-3,6-7
Y		4-5
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X	EP, A1, 0172107 (PRAXIS BIOLOGICS, INC.) 19 February 1986, see the whole document	1-4,6-7
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Y		4-5
	--	
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Y		4-5
	--	
Y	Dialog Information Services, File 155, Medline 67-91, Dialog accession no. 07152866, McGill S et al: "Membrane interactions of diphtheria toxin analyzed using in vitro synthesized mutants", & EMBO J Oct 1989, 8 (10) p2843-8	4-5
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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/NO 91/00093

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 91-09-27
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9109871	91-07-11	EP-A-	0439954	91-08-07
WO-A1- 9003437	90-04-05	EP-A-	0445128	91-09-11
		FR-A-	2636842	90-03-30
EP-A2- 0332174	89-09-13	AU-D-	3110389	89-10-12
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EP-A1- 0172107	86-02-19	AU-B-	599570	90-07-26
		AU-D-	4458885	86-02-13
		JP-T-	61502957	86-12-18
		US-A-	4808700	89-02-28
		WO-A-	86/01228	86-02-27
US-A- 4675382	87-06-23	AU-B-	573529	88-06-16
		AU-D-	1706283	83-12-02
		CA-A-	1217156	87-01-27
		EP-A-B-	0108146	84-05-16
		WO-A-	83/03971	83-11-24
FR-A1- 2532850	84-03-16	NONE		